A novel In Vitro Model for Studying Nanoparticle Interactions with the Small Intestine

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Abstract: Manufactured nanomaterials provide promising features for new technologies in cosmetic, food, and pharmaceutical applications. On the other hand, orally ingested nanomaterials/nanoparticles may interact with or enter intestinal cells via different mechanisms, resulting in possible injuries of the biological system. For that reason, the current study aims to provide useful information concerning physicochemical properties of nanoparticles with regard to cytotoxic effects and uptake mechanisms in the small intestine. Differently charged polystyrene nanoparticles were used and cytotoxicity and uptake were studied with an intestinal *in vitro* co-culture model, mimicking the villus epithelium and a triple-culture model recapitulating the follicle-associated epithelium. Mechanisms of cellular transport were investigated at 37°C and 4°C to verify that internalization mainly occurs energy-dependently. Chemical inhibitors (i.e., chlorpromazine, genistein, dynasore) were used to block dynamin-dependent endocytic pathways without affecting cell viability and membrane integrity. Qualification and quantification were performed via confocal microscopy and flow cytometry. Furthermore, co-localization studies with commonly used markers (i.e., transferrin, lactosylceramide) were carried out and co-localization was assessed via calculation of Pearson's correlation coefficient and Mander's overlap coefficient. The results show that size and surface chemistry play a crucial role in cytotoxic interactions and cellular uptake of nanoparticles (NPs). Independent of the surface charge, NPs strongly interact with intestinal mucus and are immobilized. Uptake predominantly occurs via M cells and is surface-charge dependent. Whereas negatively charged particles fail to enter cells, positive and neutral particles penetrate M cells energy-dependently. More precisely, both clathrin- and caveolae-mediated endocytosis are involved. It can be concluded that the presented system serves as a valuable tool to assess safety aspects of manufactured nanomaterials and hence, substantially contributes to nanosafety efforts.

Keywords: Caco-2 cells, goblet cells, M cells, polystyrene-nanoparticles, cytotoxicity, uptake mechanism

1. Introduction

The development of engineered nanomaterials and their marketing for application in food and consumer products, medical and diagnostic devices, as well as pharmaceutical drug delivery systems has increased significantly in recent years. During the use of these products/devices, nanomaterials intended to or may accidentally get into the oral cavity, are swallowed, and end up in the gastrointestinal tract (GIT). The GIT is a complex system, comprising various kinds of epithelia. The small intestine is thrown into folds, also referred to as villi and crypts, which increase the total surface area to 300–400 m² (Keita, 2010). Each villus contains enterocytes and mucus-secreting goblet cells, which form a firmly attached mucoid layer that protects the underlying tissue against microorganisms and pathogens (Fröhlich, 2014). Thus, in many cases, if nanoparticles (NPs) end up in the small intestine, they interact with mucus (via size filtering and/or interaction filtering effects) (Sigurdsson, 2013), are wrapped up and, as a consequence, cleared from the body. However, apart from the villus-epithelium, the follicle-associated epithelium (FAE) can be found. The FAE comprises membranous cells (M cells), located at the interface between the luminal environment and the gut-associated lymphoid tissue (Jang, 2004). They are covered with a thin layer of mucus, belong to the immune system and deliver a broad range of materials (e.g., bacteria, viruses, antigens) via transepithelial transport from the intestinal lumen directly to the underlying lymphoid tissues (Kerneis, 2000). Recently, it was shown that NP uptake in the small intestine predominantly occurs via M cells (Mahler, 2012; Antunes, 2013; Schimpel, 2014).

Basically, NPs can enter cells via passive or active mechanisms. Regarding the latter one, phagocytic and non-phagocytic pathways are involved. Unlike phagocytosis, which is restricted to specialized cells, other endocytic pathways occur in practically all cells by four energy-dependent main mechanisms: (i) clathrin-mediated endocytosis (CME), (ii) caveolae-mediated endocytosis (CvME), (iii) macropinocytosis (MP) and (iv) other clathrin- and caveolae-independent endocytic pathways (Conner, 2003; Hillaireau, 2009). CME is the most extensively studied pathway and implicates the engulfment of receptors associated with their ligands to a coated pit. Coated pit formation occurs due to polymerization of the cytosolic

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protein clathrin. The assembled vesicle (~ 120 nm) is pinched off from the plasma membrane by the small guanine triphosphatase dynamin (GTPase) and the clathrin coat is shed off. The vesicles fuse with the early endosomes, and finally primary lysosomes, which contain digestive enzymes, resulting in a degradative pathway (Kirchhausen, 2000; Takei, 2001; Rappoport, 2008; Doherty, 2009; Sahay, 2010). On the contrary, uptake can take place via caveolae (50-70 nm flask shaped invaginations at the plasma membrane coated with caveolin-1), resulting in caveosomes, which do not fuse with endosomes and thus, lysosomal degradation is prevented (Pelkmans, 2001; Pietiainen, 2004; Canton, 2012). The third endocytic pathway known to be involved in NP uptake is MP (non-selective). MP occurs via membrane protrusions, forming macropinosomes (size < 1 µm), which acidify and shrink or are recycled (Hillaireau, 2009). Finally, NPs may enter cells via passive diffusion (energy-independent) and can be found freely distributed in the cytoplasm (Porter, 2007; Carney, 2012).

However, it remains uncertain, which pathways are involved in the internalization of NPs via M cells and whether surface properties (such as the surface charge) significantly impact the uptake mechanism.

2. Methods

2.1 Preparation of the *in vitro* co- and triple-culture permeability models

Caco-2 cells (ACC169, HTB-37 clone from the German Collection of Microorganisms and Cell Cultures) were cultivated at 37°C under 10% CO₂ and water saturated atmosphere in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen GmbH, Darmstadt, Germany), 1% non-essential amino acids (NEAA) (Invitrogen GmbH, Darmstadt, Germany), 1% L-glutamine (Invitrogen GmbH, Darmstadt, Germany) and 1% penicillin and streptomycin (PenStrep) (Invitrogen GmbH, Darmstadt, Germany) according to the protocol of des Rieux *et al.* (des Rieux, 2007). HT29-MTX cells were kindly provided by T. Lesuffleur (INSERM UMR S 938, Paris, France) and were grown in the same medium under a humidified atmosphere (Lesuffleur, 1993). Raji B cells were a kind gift from R. Fuchs (Medical University of Graz, Austria). Raji B cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, and 1% PenStrep at 37°C in a humidified 5% CO₂ atmosphere. Cells were cultured as previously described (Schimpel, 2014).

For experimental studies with the triple-culture model (Caco-2/HT29-MTX/Raji B triple culture), Caco-2 and HT29-MTX were seeded at a density of 4 x 10⁴ cells/per well at a ratio

of 7:3 Caco-2:HT29-MTX onto 24-well culture plates (for flow cytometric analysis) and WillCo-dishes® (for confocal uptake visualization of intracellular particles). After 15 days, 1 × 10⁶ Raji cells/per well were added basolaterally to the co-culture and incubated for additional 4 days (100 µl of culture medium replaced every day). Raji B cells were removed immediately before NP transport studies. The model was compared with the co-culture comprising Caco-2 and HT29-MTX cells.

2.2 Histochemical characterization of M cells and mucus

M cells were identified with wheat germ agglutinin (WGA) staining following previously described protocols (Schimpel, 2014) and the samples were observed using fluorescence microscopy (Zeiss LSM 510 META) at 488 nm excitation wavelength using a 505/550 nm band pass detection (BP) for the green channel and 405 nm excitation wavelength in conjunction with BP 420/80 for the blue spectral region. Moreover, M cells show a down-regulation of the brush border enzyme alkaline phosphatase (ALP) compared with Caco-2 cells, thus, ALP activity was determined by using SIGMAFAST™ p-Nitrophenylphosphate tablets (Sigma Aldrich, Munich, Germany) according to the manufacturer's instructions. The rates of absorbance (M cells versus enterocytes), which are directly proportional to the ALP enzyme activity were determined at 405 nm and quantification was conducted with a VIS-plate reader (FLUOstar Optima, BMG, Labortechnik). In order to provide evidence of mucus and mucus-secreting goblet cells, Alcian Blue staining was performed. Briefly, cells were placed in 3% acetic acid for 3 min, followed by staining with Alcian Blue solution (1 g Alcian Blue 8GX in 100 ml 3% acetic acid). After 30 min of incubation at room temperature, cells were extensively washed twice with distilled water. Finally, cells were visualized by light microscopy (Olympus BX-51, camera: DP-71).

2.3 Particle characterization

Particle dispersions of 50 nm green fluorescence-labeled plain polystyrene (441/486) (50 nm PP), 200 nm red fluorescence-labeled plain polystyrene (542/612) (200 nm PP) particles (Fisher Scientific, Vienna, Austria), 200 nm red fluorescence-labeled aminated (542/612) (200 nm AP) particles (Molecular Probes, Vienna, Austria) and 20 nm and 200 nm red fluorescence-labeled carboxylated (20 nm CP, 200 nm CP) particles (Molecular Probes, Vienna, Austria) at a concentration of less than 1 mg/ml were prepared to determine the mean particle size and the zeta potential. The particles were suspended in MilliQ water, serum-free DMEM and phosphate-buffered saline (PBS) and ultrasonicated for 10 min. Particle size was measured via

photon correlation spectroscopy (PCS, Malvern Zetasizer, Malvern Instruments, Malvern, UK) equipped with a 532 nm laser. The zeta potential was measured by laser Doppler velocimetry (scattering angle of 17°) coupled with PCS (Zetasizer Nano ZS, Malvern Instruments) and calculated from the electrophoretic mobility by applying the Henry equation.

2.4 Cytotoxicity study and LDH release assay

To evaluate whether polystyrene NPs are harmful to the M-cell model, studies on the mitochondrial activity and on the cell membrane were performed in a similar manner as described earlier in literature (Teubl, 2013). For this, a CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega GmbH, Mannheim, Germany) was used. In the presence of the electron coupling reagent phenazine methosulfate (PMS) the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS) is bio-reduced by cells into a colored formazan product that is soluble in tissue culture medium. Cell membrane integrity was studied using CytoTox-ONE homogeneous membrane integrity assay (Promega GmbH, Mannheim, Germany). Caco-2 and HT29-MTX were seeded in a 96 well plate at a density of 2×10^4 cells at a ratio of 7:3 (Caco-2:HT29-MTX) and were cultured for 15 days. Next, Raji B cells were added and cultures were incubated for 4 days. Subsequently, the medium was replaced with PP50, PP200, CP20, CP200, AP200 particles/serum-free medium (SFM) dispersions in different concentrations (i.e., 10, 50, 100, 150, and 200 µg/ml, $n = 3$) and incubated for 4 h. 20 µl of a MTS/PMS solution per well were added and resuspended. After an incubation time of 4 h, the absorbance was measured at 490 nm with a VIS-plate reader (FLUOstar Optima, BMG, Labortechnik, Offenburg, Germany).

Briefly, for the lactate dehydrogenase (LDH) leakage assay control wells (100% LDH release) were treated with 2 µl of lysis solution. 25 µl of the supernatant were mixed with 25 µl of the CytoTox-ONE Reagent in a white microplate. After incubation, the fluorescence was recorded at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. After subtraction of blank value, the average fluorescence from the samples was normalized to the maximum LDH release (lysis control). To detect possible interferences of NPs with the MTS or LDH assay (e.g., binding of assay components, interference due to fluorescence or absorbance of NP at the same wavelength of the assay dye), interference controls (blank samples containing no cells but treated exactly the same way with NP or cell lysis buffer) were run in parallel on the same 96-well plate as recommended by Rösslein *et al.* (Rösslein, 2014).

2.5 Cellular uptake/quantification of NPs

Uptake of polystyrene NPs was assessed via confocal laser scanning microscopy (CLSM) and flow cytometry (FACS). For CLSM imaging, the co-culture and the triple culture were grown on WillCo-dishes® at a density of 4×10^4 and incubated at 37°C for 4 h with 100 µg/ml NPs. After incubation, cell nuclei were stained with Hoechst 33342 (Invitrogen, GmbH, Darmstadt, Germany). Subsequently, cells were fixed with 4% formalin in PBS and CLSM (Zeiss LSM 510 META) equipped with ZEN software package (Zeiss Germany) was performed. Red fluorescence-labeled 200 nm NPs were detected at 543 nm excitation wavelength using a LP 560 nm BP detection for the red channel, whereas 405 nm excitation wavelength in conjunction with BP 420/480 nm was used for detecting Hoechst 33342. WGA-stained cell borders were visualized at 488 nm laser excitation using a BP 505/550 nm BP detection for the green spectral region. The intensity of the laser beam and the photo detector sensitivity were kept constant to compare the relative fluorescence intensities between the experiments. NP uptake was analyzed using CLSM images from randomly chosen areas of the cell monolayers and images were captured with z-stack to record a three-dimensional data set.

Flow cytometric analysis was used to quantify the amount of NP uptake. The co-culture model (Caco-2/goblet cells) and the triple-culture model (Caco-2/goblet cells/M cells) were grown on 24-well plates at a density of 4×10^4 /per well and incubated with 100 µg/ml red fluorescence-labeled polystyrene NPs for 4 h at 37°C. After removal of the medium, the cells were washed three times with PBS. Next, cells were trypsinized to obtain a single-cell suspension, centrifuged, and cell pellets were resuspended in PBS. Finally, samples were counterstained with the dead-cell indicator SYTOX® Blue (Invitrogen GmbH, Darmstadt, Germany), which is a high-affinity nucleic acid stain that only penetrates cells with compromised plasma membranes. To calculate the background fluorescence, untreated cells were used as negative control.

Samples treated with NPs were subsequently analyzed by FACS LSR II (BD Biosciences, San Diego, US) to determine the internalized NP concentration. Viable cells (SYTOX® Blue stained–negative cells) were gated using the 525/50 channel. The results were reported as the mean fluorescence obtained by measuring 10,000 events, averaged between 3 independent replicas of 3 independent experiments.

2.6 *In vitro* cytotoxicity of endocytic inhibitors

To examine the most efficient concentrations of the distinct endocytic inhibitors without affecting cell viability, a CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega

GmbH, Mannheim, Germany) was carried out similarly as described previously (Teubl, 2013). Cells were seeded into a 96-well microplate as described before. Medium was replaced by medium containing endocytic inhibitors at various concentrations, which ranged from 33 to 140 μM for chlorpromazine, 60 to 150 μM for dynasore, and 200 to 700 μM for genistein. Cells were incubated for 4 h 40 min at 37°C in a humidified atmosphere. After the incubation period, negative controls were prepared by the addition of 2 μl lysis solution to wells containing cells only. Next, the MTS assay solution was added into each well and cells were incubated for another 4 h at 37°C in a humidified atmosphere. The optical density at 492 nm was measured by using a VIS-plate reader (FLUOstar Optima, BMG, Labortechnik, Offenburg, Germany). The viability of the cells exposed to pharmacological inhibitors was expressed as a percentage of the viability of cells grown in absence of inhibitors (i.e., 100% viable control). Cell viability greater than 80% was classified as acceptable for NP uptake experiments.

To evaluate possible negative effects on the cell membrane, a LDH assay was performed. Cells were seeded as described, the medium was replaced by the endocytic inhibitors/serum-free medium dispersions and incubated for 4 h 40 min. LDH leakage was determined using a CytoTox-ONE homogeneous membrane integrity assay (Promega GmbH, Mannheim, Germany) according to the manufacturer's specifications. Control wells (100% LDH release) were treated with 2 μl of lysis solution. The supernatant (25 μl) was mixed with 25 μl of the CytoTox-ONE Reagent in a white microplate. After 10 min of incubation (at RT), the reaction was stopped by adding 12.5 μl of stop solution. The fluorescence was recorded by fluorometer (FLUOstar Optima, BMG, Labortechnik, Offenburg, Germany) at 560 nm excitation wavelength and 590 nm emission wavelength. The measured fluorescence intensity produced was proportional to the number of lysed cells. LDH release from cells lysed with lysis solution was defined as 100% leakage, and LDH release from untreated cells as 0% leakage. LDH leakage below 5% was classified as suitable for NP uptake studies.

2.7 Endocytic inhibitor studies

To show that the inhibitor only affects the endocytic pathway of interest, transferrin was co-incubated with chlorpromazine (70 μM) and Lactosylceramide (LacCer)

with genistein (300 μM). Cells solely treated with transferrin and LacCer were utilized as positive controls. Subsequently, endocytic pathways involved in polystyrene NP uptake were investigated. For this, pharmacological inhibitors (Sigma Aldrich, Munich, Germany) that prohibit specific cellular uptake pathways were used at appropriate concentrations (see Table 1).

Cells were grown on 24-well culture plates/WillCo-dishes® (confocal and FACS, respectively) at a density of 4×10^4 . Triple cultures were pre-incubated (40 min) with pharmacological endocytic inhibitors, followed by the addition of fluorescence-labeled NPs (100 $\mu\text{g/ml}$; incubation 4 h). Cells solely treated with NPs were utilized as positive control. First, inhibition of both CME and CvME was conducted with the dynamin-GTPase inhibitor dynasore (90 μM) to evaluate whether MP or other dynamin-dependent pathways are involved. Second, 300 μM genistein was used to inhibit CvME. Finally, CME was assessed utilizing treatment with 70 μM chlorpromazine. Additionally, NP uptake studies were carried out at 4°C to determine energy-dependency of NP uptake since it has been reported that NP uptake can also occur via energy-independent processes, such as passive diffusion (Herd, 2013). All samples were visualized via CLSM.

2.8 Co-localization studies

To get deeper insights into the specific mechanisms involved, properly speaking to differ between CME and CvME, co-localization was studied with transferrin, a well-known marker of clathrin-mediated uptake (Rejman, 2004) and with LacCer, a caveolae-mediated endocytosis marker (Marks, 2005). For this, cells were incubated with the specific inhibitors; after that, particles were applied and transferrin (20 $\mu\text{g/ml}$) or LacCer (0.5 μM) was added.

For CLSM imaging, nuclei were counterstained with Hoechst 33342 (Invitrogen GmbH, Darmstadt, Germany). Red fluorescence-labeled 200 nm particles were detected at 543 nm excitation wavelength using a LP 560 nm BP detection for the red channel. Alexa Fluor 488® Transferrin and BODIPY® LacCer were visualized at 488 nm laser excitation using a BP 505/550 nm BP detection for the green spectral region. 405 nm excitation wavelength in conjunction with BP 420/480

Endocytic inhibitor	Concentration	Function
Dynasore	90 μM	Inhibition of the GTPase activity of dynamin, blocks pinching-off of clathrin- and caveolin-coated vesicles (Macia, 2006)
Genistein	300 μM	Inhibition of caveolae-mediated/lipid raft endocytosis, commonly utilized inhibitor of protein tyrosine kinase (Hubbard, 2000)
Chlorpromazine	70 μM	Inhibitor of clathrin-mediated endocytosis by depleting the plasma membrane of clathrin and adaptor proteins (Herd, 2013)

Table 1. Endocytic inhibitors used for uptake experiments

nm was used for detecting Hoechst 33342. Analyses were performed with the internal ZEN quantification software. By selecting images under 488 nm and 560 nm channels as image pair, Pearson's correlation coefficient (PCC) and Mander's overlap coefficient (MOC) were calculated from the following equations (Barlow, 2010):

$$PCC = \frac{\sum (R_i - R_{average}) \times (G_i - G_{average})}{\sqrt{\sum (R_i - R_{average})^2 \times \sum (G_i - G_{average})^2}}$$

$$MOC = \frac{\sum (R_i) \times (G_i)}{\sqrt{\sum (R_i)^2 \times \sum (G_i)^2}}$$

where R_i represents signal intensity of pixels in red channel and G_i represents signal intensity of pixels in green channel; $R_{average}$ and $G_{average}$ show the average intensities of these respective channels.

2.9 Statistical analysis

All experiments were performed in triplicates and the results were presented as mean $\pm$ standard deviation. Statistical analysis (one-way analysis of variance, ANOVA) was performed. Differences were considered to be significant at a level of $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results and Discussion

3.1 Development and characterization of the co- and the triple-culture model

In the current study, *in vitro* permeability models of the villus epithelium and the FAE designated to investigate endocytic

uptake mechanisms of NPs were used. For the co-culture, Caco-2 cells and mucus-secreting goblet cells (HT29-MTX) were treated on transwells. For the triple-culture model, Raji B cells were added to an enterocyte/goblet cell monolayer and differentiation into M cells occurred. M-cell identification was carried out with two histochemical methods. First, Alexa Fluor 488 fluorescence-labeled lectin WGA was used to selectively detect N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues, which are predominantly present on M-cell surfaces. Moreover, enzyme activity of the hydrolytic enzyme ALP was determined since ALP is reduced in M cells compared with enterocytes (Nicoletti, 2000). The results clearly showed a significant increase of the green emission due to higher lectin-binding-affinity of M cells compared with enterocytes and goblet cells (see Figure S1, Supporting Information File 1). Additionally, ALP activity in the triple-culture model was significantly decreased (12%; ** $p < 0.01$) compared with the control (Caco-2/goblet cells, 100%), implying the presence of M cells.

Since it is known that intestinal mucus strongly impacts the mobility of NPs, staining of mucus was performed. The presence of mucus was identified on top of the triple-culture cell surface, but not on the monoculture of Caco-2 cells (see Figure S2, Supporting Information File 1).

3.2 Physicochemical characterization and cytotoxicity of NPs

Size, surface charge and polydispersity index (Pdl) of the particles in different media were determined via PCS (see Table 2). In MilliQ water, dispersion was best and smallest sizes were recorded. The results showed that the mean hydrodynamic diameters and the zeta potential values were not affected by the different media used, thus agglomeration could be excluded.

	50 nm PP particles			20 nm CP particles			200 nm AP particles		
	d (nm)	ζ (mV)	Pdl	d (nm)	ζ (mV)	Pdl	d (nm)	ζ (mV)	Pdl
MilliQ water	51.2 $\pm$ 3.43	-15.7	0.036	31.2 $\pm$ 2.45	-39.6	0.141	201.2 $\pm$ 2.78	10.5	0.201
SFM*	62.9 $\pm$ 1.22	-20.1	0.311	44.5 $\pm$ 1.25	-31.7	0.164	222.8 $\pm$ 2.14	11.8	0.227
PBS	57.18 $\pm$ 1.04	-12.8	0.269	33.6 $\pm$ 1.14	-43.2	0.091	202.4 $\pm$ 1.78	8.5	0.300

	200 nm PP particles			200 nm CP particles		
	d (nm)	ζ (mV)	Pdl	d (nm)	ζ (mV)	Pdl
MilliQ water	209.0 $\pm$ 1.45	-8.7	0.044	245.1 $\pm$ 2.8	-46.6	0.0841
SFM*	205.8 $\pm$ 1.16	-17.3	0.103	304.2 $\pm$ 1.2	-47.6	0.162
PBS	211.8 $\pm$ 2.94	-2.4	0.089	268.2 $\pm$ 1.7	-28.8	0.158

*used for uptake studies

Table 2. Hydrodynamic diameter (nm), surface charge (ζ -potential) and polydispersity index (Pdl) of 50 nm plain polystyrene (PP) particles, 200 nm plain polystyrene (PP) particles, 20 nm carboxylated polystyrene (CP) particles, 200 nm carboxylated (CP) polystyrene particles, and 200 nm aminated polystyrene (AP) particles in MilliQ water, serum-free medium (SFM), and phosphate-buffered saline (PBS).

It is known from the literature that NPs may cause injuries to biological systems (e.g., multi-well nanotubes, silver NPs, silica particles) (Lewinski, 2008; Napierska, 2009; Park, 2011). Due to reducing particle size from the microscale to the nanoscale, the reactivity increases (Mayer, 2009) and associated with that, adverse effects might occur. To evaluate whether polystyrene particles penetrate the cells in a destructive or non-destructive manner, cytotoxicity studies were performed with the triple-culture model (Schimpel, 2014). To exclude NP interference with the test reagents (MTS/LDH), absorbance/fluorescence investigations were performed prior to cytotoxicity tests. The results revealed that exposure to 20 nm CP and 50 nm PP caused cytotoxic damage (as indicated by LDH release) and a decrease in cell survival (as determined by MTS) in a concentration-dependent manner. At 100 µg/ml (which corresponds to the concentration used for subsequent uptake experiments), cell viability was significantly decreased to 63.7% for 50 nm PP particles (** $p < 0.01$) and to 53.3% for 20 nm CP particles (** $p < 0.01$). Moreover, LDH release was considerably increased (10.2% for 50 nm PP particles and 15.6% for 20 nm CP particles (** $p < 0.01$), which is consistent with data reported by Fröhlich *et al.* (Fröhlich, 2012). In contrast, differently charged 200 nm polystyrene particles showed no cytotoxic response (see Figure 1) and were, thus, used for further experiments.

3.3 Cellular uptake/quantification using polystyrene NPs

NPs' physicochemical properties such as size and hydrophilicity are known to be relevant parameters that impact the uptake rate (Zauner, 2001; Verma, 2010). This is also reported for the surface charge (Huang, 2002; Mao, 2005; Harush-Frenkel, 2007). Depending on the cell type, negative particles display little or no association/internalization with the cell plasma membrane of epithelial cells compared with positively charged NPs. To verify this phenomenon also for M cells, mechanistic experiments considering three distinct surface charges were performed.

Evidence of particle internalization via M cells was obtained by using flow cytometry. After 4 h, all investigated NPs failed to penetrate the co-culture irrespective of the particles' surface charge. In the triple-culture model, uptake predominantly occurred via M cells. As expected, positively charged NPs as well as neutral particles were internalized by M cells within 4 h. The calculated uptake rate was 26.7% (** $p < 0.01$) for 200 nm PP particles and 33.2% (** $p < 0.01$) for 200 nm AP particles, respectively (see Table 3). Negatively charged particles failed to enter the cell since electrostatic repulsion from anionic sites of the plasma membrane (e.g., proteoglycans) inhibits their penetration (Harush-Frenkel, 2008). Similar findings were reported by des Rieux *et al.* (des Rieux, 2005). They investigated

Figure 1. Cytotoxicity/lactate dehydrogenase leakage of polystyrene NPs. The graph indicates the percentage of cell viability/lactate dehydrogenase release for different concentrations of polystyrene nanoparticles. The results are reported as mean $\pm$ standard deviation with $n = 3$.

	Particle uptake [%]		
	200 nm PP particles	200 nm AP particles	200 nm CP particles
Caco-2/ HT29-MTX	0.9 ± 0.02	1.2 ± 0.01	0.4 ± 0.01
Triple-cul- ture model	26.7 ± 2.1	33.2 ± 2.7	0.8 ± 0.02

Table 3. Cellular uptake/internalization of 200 nm PP, 200 nm AP and 200 nm CP particles in the co-culture and the triple-culture model

the impact of the physicochemical properties on the transport/uptake via M-cells using 200 nm carboxylated or aminated latex particles. In contrast to our results, both negatively charged and positively charged particles were taken up by M cells. However, aminated NPs were more efficiently transported than carboxylated NPs, suggesting that the presence of cationic groups enhances NP-cell surface-adsorption, resulting in a higher uptake. Moreover, the influence of particle charge on the uptake via M cells was also studied *in vivo*. It was shown that the uptake of negatively charged polystyrene particles via murine M cells was significantly decreased compared with non-ionized ones (Keegan, 2003).

Additionally, to NP uptake investigations via flow cytometry, confocal microscopy was performed with the co-culture as well as with the triple-culture model. Regarding the triple-

culture model, optical cross sections revealed that only neutral and positively charged particles were detected inside the cells in specific M cell-rich regions that were not covered with mucus (see Figure S3, Supporting Information File 1). However, in the co-culture, NPs - independent on their surface charge - were entrapped in the mucus layer and hence, failed to penetrate the underlying cells (see Figure 2). These results strongly suggest that the mucus layer presents a strong barrier for NPs, which is in agreement with findings by Crater *et al.* (Crater, 2010) and Behrens *et al.* (Behrens, 2002). Apart from that, recently published data by our group show that cell mechanics/dynamics (i.e., cell elasticity, adhesion) are also critical parameters that influence intestinal internalization/uptake of NPs (Schimpel, 2015). M cells display a smooth, mucus-free and more elastic surface due to the absence of a

Figure 2. Fluorescence microscopic z-scans of the triple-culture model (a, b, c) and the corresponding control (co-culture) (d, e, f) treated with 200 nm plain polystyrene particles, 200 nm aminated polystyrene particles and 200 nm carboxylated polystyrene particles (shown in red). Cell nuclei were stained with Hoechst (blue), cell borders were stained with Alexa Fluor 488 WGA (green). The circles indicate NP uptake/penetration into M cells, whereas arrows depict entrapped/immobilized particles in the mucoid layer.

microvilli-rich intestinal brush border, leading to a significantly higher M-cell adhesion ability compared with Caco-2 cells. As a result, particles adhere to the cell surface more rapidly. Additionally, cellular uptake is facilitated due to a higher elastic deformation of the cytoskeleton, which can be attributed to the decrease of filamentous actin. As a consequence, M cells show a higher bending elasticity of the cell membrane and hence promote the engulfment process of NPs.

3.4 Endocytic inhibitor studies and co-localization of polystyrene NPs

To elucidate whether the mechanism of M-cell internalization is only actively driven, cell experiments were performed at 4°C and at 37°C. NP that uptake was entirely inhibited at lower temperatures (data not shown), suggesting that particle internalization via M cells only occurs via energy-dependent pathways, which is in accordance with findings by other groups (Kerneis, 1997; Gullberg, 2000; des Rieux, 2005). Hence, the main pathway involved in internalization of NPs is endocytosis (Sahay, 2010). Currently, the two most well characterized endocytic pathways are CME and CvME. To

investigate if these routes are also dominant in M-cell uptake, we studied NP uptake in the presence of different endocytic inhibitors. For this, it is of paramount importance to assess the appropriate concentration of each inhibitor that on the one hand, does not harmfully affect cell viability and integrity and on the other hand, sufficiently inhibits the pathway of interest. Various concentrations of three frequently used inhibitors (i. e., dynasore, genistein, chlorpromazine) were tested (Rejman, 2004; Marks, 2005) and the effect on cell viability/integrity was studied. The inhibitor concentrations that revealed cell viability values $\geq 80\%$ and LDH leakage $< 5\%$ were classified as non-toxic and selected for the uptake experiments (results presented in Table 4 and Figure 3) (Vercauteren, 2010). Dynasore, an inhibitor that is responsible for vesicle scission during CME and CvME (Goldberg, 2010), showed a decrease of the cell viability with increasing concentrations. 90 μM was found to be acceptable with a viability $> 80\%$ and LDH leakage $< 2\%$. Genistein is a tyrosine kinase inhibitor and hinders the recruitment of dynamin 2, both known to be crucial for CvME (Parton, 1994; Nabi, 2003). Genistein exhibited no significant impact on the cell integrity, however, the cell viability decreased with increasing concentrations. A concentration of 300 μM

Endocytic inhibitor	Concentration	Cell viability [%] Mean $\pm$ SD	LDH (%) leakage Mean $\pm$ SD
Dynasore	90 μM	89.0 $\pm$ 3.8	1.8 $\pm$ 0.4
Genistein	300 μM	88.5 $\pm$ 2.7	4.8 $\pm$ 2.9
Chlorpromazine	70 μM	81.79 $\pm$ 2.4	3.6 $\pm$ 0.9

Table 4. Cell viability/lactate dehydrogenase leakage of endocytic inhibitors

Figure 3. Cytotoxicity/lactate dehydrogenase leakage of endocytic inhibitors. Cells were incubated for 4 h 40 min at 37°C with 3 endocytic inhibitors. The graph indicates the percentage of cell viability/LDH release for the different concentrations for each endocytic inhibitor. Results were reported as mean $\pm$ standard deviation with $n = 6$.

was found to be appropriate for subsequent experiments. Chlorpromazine, which inhibits clathrin-coated pit formation by a reversible translocation of clathrin and its adaptor proteins from the plasma membrane to intracellular vesicles (Wang, 1993; Vercauteren, 2010), was used at a concentration of 70 μM . Next, we evaluated the specificity of the inhibitors by co-incubating transferrin with chlorpromazine (70 μM) and LacCer with genistein (300 μM). Based on flow cytometry data, both inhibitors showed specific inhibition of transported transferrin and LacCer (i.e., CME and CvME) under experimental conditions (see Figure S4, Supporting Information File 1). Thus, the appropriate concentrations of the inhibitors were

used to study specific endocytic pathways involved in NP uptake. Cells were incubated with each inhibitor and positively charged and neutral NPs were added. The results showed that all inhibitors significantly decreased the uptake of 200 nm PP and 200 nm AP particles (see Figure 4). Incubation with dynasore, which is essential to inhibit CME and CvME, resulted in a marked decrease in the cellular uptake (90% compared with the positive control; ** $p < 0.01$). This indicates that MP and other pathways are likely to be involved to a minor extent, however, NP uptake predominantly occurs via CME and/or CvME. To differ and identify the main pathway involved, experiments were conducted with chlorpromazine

A

B

Figure 4. A: Influence of different endocytic inhibitors on cellular uptake of 200 nm plain polystyrene particles and 200 nm aminated polystyrene particles in the triple-culture model. B: Flow cytometry histograms; untreated cells were used as negative control and nanoparticles treated cells without inhibitors as positive control. Other values were compared to the control using analysis of variance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

to inhibit CME. The calculated values clearly showed that the uptake was significantly reduced independent of the surface charge (i. e., 64.4% and 67.8% for PP 200 nm and AP 200 nm compared with the control; ** $p < 0.01$). Treatment with genistein, used to hinder CvME, significantly decreased the uptake of NPs (44.9% and 50.9% for PP 200 nm and AP 200 nm compared with the control; ** $p < 0.05$). This implies that NP uptake via M cells is non-specific, more precisely that both CME as well as lipid raft/CvME are involved in the uptake of 200 nm polystyrene particles. He *et al.* (He, 2013) showed that the uptake of 80 nm polymeric NP in Caco-2 cells also occurs via non-specific endocytic mechanisms (i.e., 35% clathrin-dependent uptake, 50% caveolae-mediated endocytosis and 50% macropinocytosis). However, uptake of 200 nm carboxylated polystyrene NPs by M cells is reported to mainly occur by the transcellular route, predominantly via macropinocytosis (des Rieux, 2007). In contrast, our results indicate that uptake of positively and neutrally charged NP via

M cells dominantly occurs via CME and CvME. This suggests that NP surface charge is likely to influence the uptake mechanisms via M cells.

Due to electrostatic interactions between NPs and the cell plasma membrane, the usage of flow cytometry as analytical tool to reveal cellular uptake is sometimes difficult and has to be verified. One convenient way to conclude that the decrease in particle uptake in the presence of endocytic inhibitors simply arises due to their inhibiting effects (and that NPs are not just bound on the cell surface), is to perform co-localization studies with commonly used fluorescent-labeled intracellular endocytic markers, such as transferrin and/or LacCer. However, recently, it was reported that the efficacy of these markers is cell line dependent (Vercauteren, 2010). In this study, the triple-culture model was treated with NPs and transferrin or LacCer. The z-stacks clearly showed that without the addition of endocytic inhibitors, transferrin and LacCer were internalized by the cells (Figure 5). NPs were

Figure 5. Fluorescence microscopic z-scans of the triple-culture model treated with 200 nm plain polystyrene particles and 200 nm aminated polystyrene particles (shown in red) in the presence of endocytic inhibitors. Cell nuclei were stained with Hoechst (blue), endocytic markers (i.e., transferrin and LacCer) were depicted in green. The circles indicate nanoparticle uptake/penetration, whereas arrows reveal numerous yellow spots indicating the co-localization of internalized particles with transferrin (a, d) and LacCer (b, e).

detected as yellow spots in M cells (due to the overlap of green and red fluorescence) indicating co-localization with either transferrin or LacCer. The addition of dynasore entirely inhibited the uptake of both markers.

To quantify these results, co-localization was calculated by using PCC and MOC. PCC is a standard measure in pattern recognition to assess co-localization by means of describing the relationship/correlation of two fluorescent intensity distributions from two images (Zinchuk, 2008). It generates a range of values from +1, indicating a perfect positive correlation (i.e., all pixels are in straight line in the scatter diagram), to -1, a perfect but inverse correlation. Zero represents a random distribution representing no co-localization (i.e., pixels in scattergram distribute in a cloud with no preferential direction). The PCCs are classified from 0.5 to 1.0, indicating co-localization and from -1.0 to 0.5, indicating absence of co-localization. MOC is a co-localization coefficient that is insensitive to the limitations of fluorescence imaging, like photobleaching or camera quantum efficiency, just to mention two examples (Manders, 1993). MOC generates only positive values, thus, direct interpretation regarding the degree of overlap is possible. These values are in the

range from 0 to 1.0. For example, an overlap coefficient of 0.75 reveals that 75% of its both objects (i.e., pixels) overlap and three-quarters co-localization occurs - compared with zero, meaning no co-localization. Classification ranges from 0.6 to 1.0, indicating co-localization and from 0 to 0.6, revealing absence of co-localization.

The results are presented graphically in scatterplots (Figure 6A). The intensity of green colored transferrin and LacCer were plotted against the intensity of red-labeled polystyrene particles. Co-localizing pixels are shown in scatter region 3. In contrast, the lack of co-localization is reflected by the distribution of points into two separate groups, that is, scatter region 1 and 2. Co-localization of endocytic markers and NPs was apparent as yellow spots due to the overlay of green and red fluorescence.

All data from the co-localization coefficients are summarized in Figure 6B. The results revealed that for 200 nm PP particles, the calculated PCC was 0.66, indicating co-localization of the NPs with transferrin. The MOC value was 0.85, meaning an overlap of 85%. The degree of co-localization for LacCer was found to be lower, with an estimated PCC of 0.58 and MOC of 0.7. These results suggest that CvME is involved in PP

Figure 6. Intracellular trafficking of 200 nm plain polystyrene particles and 200 nm aminated polystyrene particles. **A:** Co-localization images and scatterplots of 200 nm plain polystyrene particles and 200 nm aminated polystyrene particles with CME marker transferrin and CvME marker LacCer after 4 h. Yellow fluorescence spots displayed the co-localization of NPs with transferrin and LacCer in merged images. **B:** Comparison of quantitative co-localization of 200 nm plain polystyrene particles and 200 nm aminated polystyrene particles with CME and CvME marker by examining Pearson's correlation coefficient (PCC) and Mander's overlap coefficient (MOC).

particle uptake, but less than CME. Regarding 200 nm AP particles, similar values were obtained. For all calculations background correction in the region, which was used for calculation of the correlation coefficients, was performed. This correction is important because confocal microscopy is characterized by low signal to noise ratio. An intact (not corrected) background can reach up to 30% of the maximal fluorescence intensity and cause 30% of false positive colocalization (Landmann, 2004).

In summary, the results corroborate that uptake of positive as well as neutral NPs into M cells mainly occurs via CME and less via CvME, confirming the results obtained via flow cytometry and confocal microscopy.

4. Conclusions

The current study shows that size and surface chemistry play a crucial role in cytotoxic interactions and cellular uptake of NPs. Regarding the villous epithelium (enterocytes, goblet cells), which is entirely covered with mucus, 200 nm particles interact, independent on their surface charges, with intestinal mucoglycoproteins being immobilized and, as a consequence, cleared from the body. Regarding the FAE, we observed that uptake of NPs predominantly occurs via M cells due to higher adhesion capability, an enhanced elasticity of the cell membrane and a thinner or absent mucus layer. Negatively charged particles failed to enter M cells. In contrast, positively charged NPs as well as neutral particles were internalized via active transport mechanisms, more precisely, via CME and CvME.

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Taken together, the presented data suggest that although the mucus layer is thinner compared with *in vivo* conditions, the intestinal *in vitro* models are appropriate to study interactions as well as transport mechanisms within the small intestine on a fundamental basis. Hence, the presented model can be used as an evaluation tool to assess safety aspects of manufactured nanomaterials. In addition, further tests will be performed using standard operating procedures (e.g., OECD-testing guidelines as recommended by NANoREG-project) (OECD) evaluating various manufactured nanomaterials (considering the physicochemical properties). These data will then be processed into ISA-TAB-nano-templates (ref. to eNanoMapper, NanoSafetyCluster-Modelling-projects), used for data modeling, grouping, risk categorization, and cross reading. It can be concluded that by this future outlook, the presented study substantially contributes to the nanosafety efforts to proactively show evidence of manufactured nanomaterials safety.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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